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ALTERED TNF-ALPHA, GLUCOSE, INSULIN AND AMINO ACIDS IN ISLETS OF
LANGERHANS CULTURED IN A MICROGRAVITY MODEL SYSTEM

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Abstract

The present studies were designed to determine effects of a microgravity model system upon lipopolysaccharide (LPS) stimulated tumor necrosis factor alpha (TNF-alpha) activity and indices of insulin and fuel homeostasis of pancreatic islets of Langerhans. Islets (1726 ± 117 , 150 u IEU) from Wistar Furth rats were treated as: 1) HARV (High Aspect Ratio Vessel cell culture), 2) HARV plus LPS, 3) static culture, 4) static culture plus LPS. TNF-alpha (L929 cytotoxicity assay) was significantly increased in LPS-induced HARV and static cultures, yet the increase was more pronounced in the static culture group ($p < 0.05$). A decrease in insulin concentration was demonstrated in the LPS stimulated HARV culture ($p < 0.05$). We observed a greater glucose concentration and increased disappearance of arginine in islets cultured in HARVs. While nitrogenous compound analysis indicated a ubiquitous reliance upon glutamine in all experimental groups, arginine was converted to ornithine at a two-fold greater rate in the islets cultured in the HARV microgravity model system ($p < 0.05$). These studies demonstrate alterations in LPS induced TNF-alpha production of pancreatic islets of Langerhans, favoring a lesser TNF activity in the HARV. These alterations in fuel homeostasis may be promulgated by gravity averaged cell culture methods or by three dimensional cell assembly.

Keywords: tumor necrosis factor alpha, cytokines, insulin, diabetes, microgravity

INTRODUCTION

Factors which modulate glucose tolerance and hyperglycemia in diabetes, and its related syndromes include pancreatic islet insulin secretory function, hepatic and peripheral insulin sensitivity, and glucose effectiveness (7, 42, 16). An intriguing candidate peptide which has recently been implicated in the glucose intolerance of diabetes and obesity, is the cytokine tumor necrosis factor-alpha (TNF-alpha). TNF-alpha is a potent modulator of insulin sensitivity in adipocytes (1, 13). Increased TNF-alpha induces peripheral insulin resistance which can decrease glucose utilization. While it was once believed that macrophages and adipocytes were the primary sites of TNF-alpha production, we have recently demonstrated that purified pancreatic islets of Langerhans are a potent source of TNF-alpha (20). These observations suggest a putative islet-linked site of action for TNF-alpha in glucose intolerance.

Prolonged bedrest has long been utilized as a paradigm for insulin resistance and glucose intolerance; it also resembles metabolic alterations observed in microgravity (29). Human bedrest studies (21, 22) demonstrate increased peripheral tissue insulin resistance and reduced glucose tolerance. Similarly, post-flight investigations of cosmonauts (12) after free-fall orbit demonstrate increased plasma glucose and decreased glucose tolerance. Other space-flight investigations illustrate altered C-peptide excretion (33) and insulin resistance (8) during earth orbit. These observations and others (24) suggest that bedrest and in-flight experiments support similar changes to that observed in the glucose intolerance of pre-diabetes, aging, physical inactivity or obesity.

A series of in vitro studies illustrate altered immune cell cytokine activity in microgravity which may favor increased insulin resistance via altered TNF-alpha release. The SL-3 mission demonstrated that immune responses may be altered by space flight (32). On STS-56, MC3T3-E1 osteoblasts activated in microgravity utilized less glucose and had reduced prostaglandin E₂, a proposed regulator of cytokine production (15). As little as eight seconds of microgravity has been demonstrated to alter macrophage responses (2). Lipopolysaccharide (LPS) activated macrophages secrete more IL-1 and TNF-alpha when stimulated in microgravity than on earth (6). These space flight studies may provide insight into a mechanistic link between immune modulations and glucose tolerance. However, serious questions remain as to which observations

are due to stressors of lift-off and re-entry, and which are true microgravity effects.

While there is disagreement as to whether ground-based paradigms truly simulate microgravity (38), certain cell culture systems provide a model system for investigating altered gravity effects. One such microgravity model system is the High Aspect Ratio Vessel (HARV), developed at the Johnson Space Center. The HARV is a self contained horizontally rotating cell culture system that allows for diffusion of oxygen and carbon dioxide across a semi permeable membrane. The HARV demonstrates a very low shear stress (0.5 dynes/cm^2) for one or two mm cellular aggregates (38). It has a time- averaged gravity vector of 10^{-2} g (31) as compared to that of near-earth free-fall orbit which is $10^{-4} - 10^{-6} \text{ g}$. Thus, the HARV is a useful paradigm for studying cellular physiology in a ground based, cell culture system, which demonstrates both low shear stress and a gravity averaged free-fall paradigm.

The present studies were designed to determine the main and interactive effects of microgravity and addition of LPS on TNF- α activity, insulin secretion and glucose concentrations in cultured islets of Langerhans. An additional aim was to determine alterations in amino acid and nitrogenous compound utilization as an explanation for altered glucose concentrations in the microgravity model system.

SUBJECTS AND METHODS

Animal Care

Male Wistar Furth rats (Harlan Sprague-Dawley, Houston TX, Indianapolis, IN) were obtained at 9-10 weeks of age, approximately 220 grams body weight, and housed in shoebox cages ($n=3$ / cage) with cellulose bedding. Animals were maintained on a twelve hour light/dark cycle (lights on 0700 hr) and had access to food and water ad libitum. All procedures were carried out in accordance with the guidelines of the National Institutes of Health and were approved by the NASA-Johnson Space Center and Mercer University Institutional Animal Care and Use Committees.

Study Design

There were two studies carried out in these experiments (Fig 1a): In Study A, we

contrasted the HARV cell culture technique vs. the static plate paradigm. In Study B the previous groups were further subdivided into LPS-stimulated and non-stimulated treatment groups. This experimental design allowed us to differentiate the independent effects of the cell culture method (HARV vs. PLATE) under basal conditions upon TNF-alpha activity, glucose, insulin, lactate and amino acids (Study A), and, determine if interactions of LPS administration and the cell culture method were operative in regulating glucose and insulin levels (Study B).

Pancreatectomy and Islet Isolation: Day 1, 2

All animals were acclimatized to the laboratory for one to two weeks prior to beginning experiments. We removed food from the cages of donor male Wistar Furth rats at approximately 1800 h; at 0800 h the following morning (day 1, Fig. 1a) the rats were anesthetized with 55 mg/kg sodium pentobarbital i.p., and pancreatectomized. Animals were exsanguinated under surgical anesthesia following pancreatectomy. Islets were isolated using established methods (3), with modifications previously described (11). Purified islets were hand picked to obtain approximately 1700 islets per treatment group, using 25x magnification with a green illuminated background and white fiber optic side illumination.

Static Culture of Islets Following Islet Isolation: Day 1, 2

Freshly isolated islets of Langerhans were placed into Medium-199 (with additional 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum, 25 mmoles/l Hepes buffer, 0.68 mmoles/l L-glutamine) as previously described (11). Islets were isolated over a two day period. The islets from two rats each (6 rats per day of isolation), were placed into 6-well polystyrene culture plates with 10 ml Medium-199 per well (approx. 1200 islets). Islets were introduced into static culture at approximately 1700 hrs. Islets remained on static culture for 24 - 48 hours prior to being aliquoted into HARV cultures for experiments

HARV Cell Culture Techniques for Pancreatic Islets: Day 3, 4

Islets were aspirated from culture plates and cells were transferred into one 50 ml conical vial and brought to 40 cc with M-199. Islets from all pancreatic isolations were mixed, then

aliquoted into 4, 15 ml conical vials and centrifuged at 1000 rpm. The vials were vortexed, and aliquots of 100 μ l (3x) were removed for determination of islet diameter, which was estimated according to standards established by Ricordi and colleagues (30). A one ml sample was obtained and designated as 0 HRS (Study A) and frozen at -70° C.

Islets (1726 ± 117 , 150 u IEU) were aliquoted into 2, 10-ml High Aspect Ratio Vessels (HARV, Synthecon, Houston) and into culture plate wells and were placed into a 5% CO₂, 95% humidified, 37° C incubator. The initial HARV speed was set at approximately 12 revolutions per minute. All bubbles were carefully removed from the HARVS prior to initiating revolutions.

Preparation for LPS Stimulation: Day 5

Following 48 hours of HARV or static culture, 1 ml of islet media was aliquoted for analysis and designated as 48 HRS (Study A). Both HARV and static cultures had 5 ml freshly prepared Medium-199 added to bring the total volume to 10 ml. All subsequent samples were taken by isovolumetric techniques. In the HARV, this was accomplished by infusing 300 μ L of cold (4° C) Medium-199 into the bottom port of a vertical, stationary HARV while removing 300 μ L of sample from the upper port. A similar technique was used in static cultures to assure equal treatment perturbations.

LPS Stimulation: Samples for TNF, Insulin, Glucose, Lactate: Day 5, 6

Prior to LPS addition, a 300 μ L sample was removed from all treatments and designated as 0 HRS post LPS stimulation. At time zero, 100 μ L of a 100 μ g/ml LPS (EColi 026:B6, Sigma, St. Louis) dissolved in Medium-199 were added to HARVs and static cultures. Islet medium samples were obtained (300 μ L) for TNF, glucose, insulin, and lactate analysis at 3, 6, 12, 24, and 48 hours post LPS stimulation. The samples were placed immediately into 1.8 ml cryovials and stored at -70° C for subsequent analyses.

Islet and Cell Image Analysis: Days 3, 5 (cohort group)

Islets were obtained from HARV and static cultures for image analysis by phase contrast and confocal microscopy. Representative images islets of Langerhans from the initial static

culture period (day 3), and islet cells cultured in HARVs (day 5) were obtained on a Hund Willovert S inverted microscope equipped with phase contrast optics, a green filter, and white fiber optic side illumination. Islets were maintained in culture medium and images were acquired using a Cannon EOS 1N 35mm camera and a 40x air objective.

Sample Analysis

Tumor Necrosis Factor Assay: The L929 mouse fibroblast assay, as previously described (18, 19, 20), was used to measure islet medium TNF activity. Briefly, L929 cells were grown to confluence overnight in 96 well culture plates; Actinomycin-D (5 µg/ml; Merck Sharp & Dohme, Weston, PA) was added to each well and serial dilutions of experimental islet medium were added to duplicate L929 wells. Following incubation, adherent L929 cells were stained with 0.5% crystal violet, optical density of each well was spectrophotometrically measured and % cytotoxicity of L929 cells was determined (27). TNF activity was then converted to Units/ml (U/ml); one unit of TNF activity was defined as equal to 50% L929 cytotoxicity in the appropriate dilution of islet medium. Dose-response inhibition of TNF activity using rat-specific TNF-alpha antibodies (Endogen Inc., Boston, MA) were performed on islet medium samples to confirm that L929 cytotoxicity was due to TNF-alpha activity.

Insulin Analysis: Islet medium was diluted 1:500 in Medium-199, and insulin was determined by competitive binding radioimmunoassay (Linco, St. Charles, MO) with antibodies raised against rat insulin and using rat insulin standards (25). Prior determination of the within-assay coefficient of variation for 8 assays of pooled rat plasma was estimated at 6.2%.

Glucose and Lactate Analysis: Samples were analyzed in duplicate as is or diluted 1:10 with distilled water for glucose and lactate analysis using a YSI 2300 Stat Plus Analyzer (Yellow Springs Instruments, Yellow Springs OH).

Amino Acid / Metabolite Assay: Analysis of nitrogenous metabolites were analyzed post-hoc to probe an explanation for altered glucose and lactate data in Study A. There was insufficient sample for amino acid analysis of Study B. The cell culture media (400µl) was prepared for analysis by precipitating protein from the samples with an equal amount of sulfosalicylic acid solution (Pickering Seraprep) and centrifuging. The supernatant (300µl) was

aliquoted and an internal standard (Glucosaminic Acid, Sigma Chemical) was added. The sample was filtered (0.2 μ m) and placed on the amino acid analyzer (Beckman 6300).

The Beckman System 6300 High Performance Amino Acid Analyzer uses ion exchange chromatography with post-column ninhydrin reaction and visible colorimetric detection for the analysis of amino acids. Depending on their dissociation characteristics, the amino acids are differentially eluted from the column with a series of lithium buffers of increasing pH and ionic strength, and further positioned by increasing the column temperature during the run. Ninhydrin, mixed continuously with the column eluent, reacts in a highly specific manner with the separated amines to form colored products, the intensity of which is proportional to the concentration of the amino acid present.

Lactate Dehydrogenase (LDH): LDH assays were performed in the supernatant of all samples from Study B, using an in vitro toxicology assay (Sigma TOX-7, St. Louis, MO).

Statistical Analysis: There were multiple replications of the experimental protocol in which two HARVs and two static plates were studied concurrently. In the initial studies, five replications (islets from 12 pancreases) resulted in n=10 per experimental treatment group for TNF-alpha, insulin, glucose, lactate analysis and amino acid analysis. Study B contained a subset of n=4 different islet populations per group throughout. The experimental paradigm included dependent variable analysis consisting of: A) basal TNF, insulin, glucose, lactate and nitrogenous analytes during 48 hours of cell culture, and B) LPS stimulation of TNF-alpha secretion, and subsequent insulin, glucose, and lactate measures during 48 hours of cell culture. The independent variables used for statistical analysis included: METHOD (HARV or static PLATE culture), LPS (LPS stimulated, or non-LPS stimulated), and TIME (0, 48 hours for Study A; 0, 3, 6, 12, 24, 48 hours for Study B).

Study A, was analyzed by two-way (between | within) repeated measures analysis of variance with interaction (GLM, METHOD vs TIME, SAS ver. 6.11, Cary NC, Snedecor and Cochran 1989). Post-hoc differences between treatments within a time period, were determined by a post-hoc Duncan's test. A value of $p < 0.05$ was considered as statistically significant. Means with similar superscripts are not significantly different by post-hoc analysis ($p > 0.05$).

Study B, was analyzed by three factor (between | between | within) repeated measures

ANOVA (METHOD vs LPS vs TIME); main effects and interactions were determined. Raw data was used for all analyses with the exception of the insulin data. Due to a twofold greater basal insulin concentration in experiment 4, all data were transformed and normalized to % baseline prior to statistical analysis. At discrete time points, a one-way between-subjects analysis of variance was performed, and a post-hoc Duncan's test was used to determine statistical significance between groups ($p < 0.05$). Means with similar superscripts are not significantly different by post-hoc analysis ($p > 0.05$).

RESULTS

Pancreatectomy and Islet Isolation

Islets were isolated from living pancreas donors and were placed into cell culture within 5 hours following pancreatectomy. Five complete experiments were performed on separate islet populations, with four treatment groups per experiment for Study A; a subset of four islet populations per treatment were studied in Study B. There were no significant differences for the 150 micron islet equivalent mass in the islet populations between experimental groups ($p > 0.05$, Fig 1b). The average number of islets per treatment group, was 1726 ± 117 islet equivalent units. All values as shown in figures are mean \pm SEM.

LPS Stimulated-TNF-alpha Production

Prior to LPS stimulation there were no significant differences in L929 TNF-alpha cytotoxicity for any treatment group in any statistical measure (Fig 2a).

Following stimulation with LPS there were significant increases in TNF-alpha of both static and HARV cultured cells (Fig 2b). Three-way repeated measures ANOVA indicated significant between-subjects effects for METHOD ($p = 0.0049$, static culture $>$ HARV), LPS ($p = 0.0001$, LPS $>$ non-LPS), and HARV*LPS ($p = 0.0046$, static culture LPS $>$ HARV LPS). Within-subjects effects were significant for TIME ($p = 0.0001$) and an interaction of HOURS*LPS ($p = 0.0001$). Islets stimulated with LPS in static culture retained a significantly greater TNF-alpha concentration vs LPS stimulated HARV cultures throughout the entire 48 hours of the study. There were no significant changes in TNF-alpha among control cultures.

Insulin Concentration

At time zero, insulin concentrations were less than 100 nmoles/l, and were not significantly different between HARV and static culture treatment groups (Fig 3a). Repeated measures ANOVA indicated a significant effect of TIME ($p=0.0004$, 48 HRS > 0 HRS). Following 48 hours of culture, there was greater than a 3-fold increase in immunoreactive rat insulin concentrations. However, there were no significant differences between HARV and static cultures at 48 hours.

There was a marked linear increase in the insulin concentration of both HARV and static culture islets which were not stimulated with LPS (Fig 3b). Repeated measures ANOVA indicated no significant differences between-subjects; however, within-subjects effects of HOURS and HOURS*LPS were significant ($p=0.0001$, $p=0.0001$). In both HARV and static cultures which were stimulated with LPS, insulin secretion was attenuated relative to non-stimulated treatment paradigms. At 48 hours post-stimulation with LPS, both control groups were different from the HARV-LPS group. However, the static plate culture islets with LPS, although intermediate in value at 48 HRS, did not differ from any other group in their insulin content.

Glucose Concentration

Prior to cell culture, there were no significant differences in the amount of glucose in islet medium between HARV or static culture groups (Fig 4a). Two-way repeated measures ANOVA demonstrated a significant interaction of Time and Method ($p=0.0488$). The media from islets in the PLATE culture had the lowest glucose concentration at 48 hours ($p<0.05$).

Following stimulation with LPS, there were relatively minor differences in the media glucose concentration up to 12 hours of culture (Fig 4b). Repeated measures ANOVA indicated a significant effect of METHOD ($p=0.0203$, HARV > static culture). The within-subjects effect of HOURS was also significant ($p=0.0026$). At 48 hours post LPS stimulation, the static culture group had metabolized the greatest amount of glucose from the media. The highest media glucose concentrations were observed in the non LPS-stimulated HARV cultures, which were

significantly different from the LPS stimulated static culture islets.

Lactate Concentration

Media lactate concentrations increased during the initial 48 hour culture period to a level which was significantly different from the zero hour value (Fig 5a). Repeated measures ANOVA illustrated a significant effect of TIME ($p=0.0001$, 48 HRS > 0 HRS). However, there were no significant differences between the lactate concentrations of HARV or static culture groups.

During the LPS stimulation studies, all treatment groups increased their lactate concentrations (Fig 5b). Repeated measures ANOVA indicated a significant main effect only for TIME ($p=0.0001$). At 48 hours of study, there were no significant differences between groups.

Amino Acid / Nitrogenous Metabolite Concentrations

Amino acids and nitrogenous analytes were arranged into functional groupings and are depicted in Table 1. Urea cycle metabolites of arginine and ornithine both demonstrated an equimolar bidirectional shift in concentration favoring a greater difference in HARV (TIME*METHOD, $p=0.0027$, 0.0001). Urea concentrations increased over time (Within, Time, $p=0.0001$) as did NH_3 , which was equimolar in both HARV and PLATE (Within, Time, $p=0.0001$).

Nitrogen transfer metabolites illustrated that glutamate production, and glutamine utilization was equimolar in both HARV and PLATE (Within, Time, $p=0.0001$ for both). Alanine concentrations increased in the islet media during the 48 hours of study (Within, Time, $p=0.0223$).

Neurotransmitter synthesis metabolites of gamma-aminobutyric acid and phosphatidylserine increased with time (Within, Time, $p=0.0001$). Glycine, an amino acid associated with creatine, heme, and purine biosynthesis increased with time in both HARV and PLATE (Within, TIME, $p=0.0341$).

Lactate Dehydrogenase (LDH)

There were no significant differences in LDH values at any time point in Study B (Table

2). Repeated measures ANOVA with interaction determined significant between-subjects overall effects of METHOD (HARV<PLATE, $P=0.0505$). Within-subjects analysis was significant for TIME only ($p=0.001$).

HARV Generated Islet Cell Aggregates (ICAs)

Phase contrast microscopy of isolated islets of Langerhans cultured on static plates for 24-48 hours, illustrates intact islets with apparently intact collagen capsules, as well as some freely circulating cells from islets which were over digested (Fig. 6a). Following forty-eight hours of HARV culture, cellular aggregation is apparent as a ubiquitous phenomenon in ICAs cultured in the microgravity model system (Fig. 6b). A prominent capsular appearance in the periphery of the matrix of ICAs is apparent.

DISCUSSION

The present studies were designed to investigate the influence of the HARV microgravity model system and LPS administration upon TNF-alpha production of pancreatic islets of Langerhans and elucidate related changes in insulin and glucose homeostasis. Studies were designed to determine the independent influence of cell culture method and LPS stimulation upon TNF-alpha activity and hormonal production, and elucidate potential interactions between these variables. Additionally, investigation of nitrogenous analytes were performed to elucidate an explanation for glucose and lactate data. The current studies demonstrate five novel observations: 1) LPS induced TNF-alpha production is lesser in the HARV islet media, than in static cultures, 2) the increase in TNF-alpha is associated with a lesser basal insulin secretion especially in HARV cultures, 3) islets cultured with the HARV are associated with a lesser glucose consumption, than are islets from static culture, 4) HARV islets demonstrate an equimolar increase in the utilization of arginine and production of ornithine, and 5) islets cultured with HARVs, will aggregate into "tissue like units", which we will designate as "Islet Cell Aggregates" (ICAs).

A most interesting and novel result in our present study is that isolated islet preparations in HARVs had increased TNF levels with exposure to LPS indicating that TNF-producing cells are present in ICAs and that LPS stimulates TNF secretion in these islets. Our present studies indicate possible involvement of TNF at the site of insulin secretion, the pancreatic islets of rats. Others have suggested that TNF gene expression may be located in the pancreas and may play a role in islet function. Norman et al. (28) noted increased TNF messenger RNA expression and intrapancreatic TNF detectable 1-6 hours after onset of acute pancreatitis induced by infusion of a cholecystokinin analogue into mice. They attributed this TNF expression to acute infiltration of macrophages during the pancreatic inflammation. Toyoda et al. (37) detected TNF-alpha in mouse islets during development of diabetes in the non-obese diabetic mouse strain NOD-Sansum. They noted that fewer than 1% of islet immune cells in this strain were macrophages and suggested that TNF-alpha may be produced by islet T cells during an autoimmune reaction in the islets of this diabetic mouse. Our data provides further evidence that islets can be sites of TNF secretion in rats. We believe this is an intriguing observation with respect to islet secretory

function. The lesser LPS induced TNF-alpha production in the HARV was not expected, and is contrary to that which has been previously observed during in-flight studies of LPS stimulated macrophages (6). The present investigations suggest three possibilities: 1) that islets behave differently from macrophages following LPS stimulation in a microgravity model system, 2) that results obtained in the HARV are not directly comparable to in-flight experiments, or 3) that the aggregation of cells into ICAs differentially modulates the production of TNF-alpha, following stimulation with LPS. The direct applicability of these data to in-flight studies remains to be determined.

That TNF-alpha is associated with a decreased basal insulin secretion is intriguing, both as it relates to in-flight extrapolations, ground based bed rest models, and as it suggests insight into the pathophysiology of Type 1 and Type 2 diabetes mellitus. In-flight studies during the Skylab mission (17) illustrated a consistent decrease in the plasma insulin concentration from 38 to 82 days. A decrease in pancreatic insulin secretion is seen both following autoimmune insulinitis of type 1 diabetes (10) and following prolonged peripheral insulin resistance with compensatory pancreatic-derived hyperinsulinemia in type 2 diabetes (39). It is not possible to say if these data are comparable, as serum insulin values are affected by rates of insulin secretion, insulin sensitivity, and insulin clearance: factors which were not measured on Skylab. In addition, plasma glucose values were also decreased in Skylab crewmembers, suggesting improved glucose control. However, the Skylab data have been viewed as conflicting, since a sharp drop in insulin was followed with a trend towards an increase, which was subsequently followed weeks later by a spike in insulin during the third and fourth weeks of flight (26). This could occur if there were a compensatory increase in insulin production secondary to reduced insulin sensitivity as suggested by Stein (33); however, this would have to occur in a time-dependent process with pancreatic function reacting secondarily and/or primarily to spaceflight stressors. The present studies raise the possibility of an endogenously mediated decrease in insulin secretion, as a secondary consequence of altered cytokine production in the HARV microgravity model system. That a lowest basal insulin secretion was observed in LPS stimulated HARV cultures, is a novel observation, and suggests that islet derived TNF has a potent capacity to down regulate islet insulin secretion in microgravity model systems. We hypothesize that this reduction in insulin

secretion occurs at a lower media concentration of TNF-alpha in the HARV, than is observed in the static plate controls, possibly due to higher intracellular concentrations of TNF in the ICAs. This hypothesis is currently under investigation in ongoing studies.

Ground based-bed rest studies support the hypothesis of a progressive increase in insulin resistance, which is accompanied by a decrease in muscle mass following prolonged physical inactivity (4). TNF has not been related to this effect, but has been suggested as a mechanism of obesity induced insulin resistance, which may accompany decreased activity levels (1, 14). Others have suggested that TNF may play a direct role in islet cell function during the autoimmune response in the development of insulin-dependent diabetes mellitus. Dunger et al. (9) found that direct TNF exposure inhibited insulin secretion and caused significant DNA strand breakage in isolated rat islets. Others reported that TNF attenuated islet cell function and proposed that direct stimulation by TNF may be involved in modulation of insulin secretion from alpha cells during the progressive autoimmune development of insulin-dependent diabetes mellitus. The pathophysiology of Type 2 diabetes mellitus is known to involve a decrease in insulin sensitivity, an initial compensatory hyperinsulinemia, followed by a subsequent decline in pancreatic insulin secretion (7). When sequentially combined, these events promulgate hyperglycemia. The influence of TNF-alpha in peripheral insulin resistance of adipose tissue has been implicated in the pathophysiology of Type 2 diabetes (13, 14). The present studies provide an additional mechanism by which islet derived TNF-alpha may contribute to the development of Type 2 diabetes by suppressing insulin secretion and promulgating hyperglycemia. The clinical relevance of these observations is unknown. The consistent increase in media insulin concentration, and the similar LDH activity of all groups, demonstrates two measures of cell viability throughout the study.

It has been proposed that insulinitis with inflammatory cell influx into islets is responsible for any islet expression of TNF which may then influence islet function. While macrophages are present in islets and may be producing TNF in response to LPS, we propose that other possible sites of TNF production may also exist in the islet infrastructure. The many endocrine cell types located in islets could be possible sources of TNF, but vascular smooth muscle cells of the complex intra-islet vasculature may also be secreting TNF. In previous studies (27), we have

shown that human blood vessels can be a significant source of TNF. We found that when stimulated with LPS, the time-dependant release of TNF from human vascular tissue was significantly increased compared with time-matched non-stimulated control vascular tissue. In human smooth muscle cells cultured from both internal mammary arteries and saphenous veins, the release of TNF into the medium essentially mimicked that seen in the intact vascular segments. Our experiments showed that TNF release occurred from both intact blood vessels and smooth muscle cells. Collectively, these findings suggested that at least one source of TNF may be the smooth muscle cell within vascular tissue. Since all islets have afferent arterioles that branch into numerous capillaries to form glomerular-like structures which then form an extensive network of peri-insular collecting venules, it is an intriguing possibility that cells, other than macrophages, located in or near such highly vascularized islet beds may also synthesize and secrete TNF. Preliminary immunohistochemical studies (data not shown) illustrate that TNF-alpha is present in the cytosol of endothelial cells of the islet vasculature, and the cytosol of islet endocrine cells following LPS stimulation; TNF-alpha is not present in exocrine tissue. Thus, the exact sites of TNF-alpha gene transcription and translation remains to be delineated in future studies.

The present studies demonstrated alterations in disappearance of glucose and arginine, and differences in appearance of ornithine as a result time and the cell culture method used. The lack of a change in media glucose concentration of HARV cultures during 48 hours of Study A, and 48 hours of LPS culture in Study B, suggests that alternative fuel sources have been utilized or that basal energy expenditure was significantly reduced in the microgravity model system. The equivalent values in lactate concentration between groups over time indicate this phenomenon is not explained by altered glycolytic activity. In the basal state 30% of islet metabolic energy requirements are met by the oxidation of the amino acid glutamine (23). Glutamine is the most abundant amino acid in the body and plays a primary role as a carrier of nitrogen between organs, with it's amide group used for nucleic acid biosynthesis (41). We added 0.68 mmol/L L-glutamine to the Medium-199 utilized in the present studies, an amount sufficient to support basal energy expenditure. The nitrogenous compounds analyzed in the current experiments illustrate that glutamine is used ubiquitously by islets of Langerhans,

regardless of the experimental paradigm. The equimolar increase in alanine in HARV islets is consistent with the concept of an increased utilization of glutamine for energy by the splanchnic organs, in times of catabolic stress. However, arginine disappearance and ornithine appearance coexist with a 2x greater change in urea concentrations in HARVs. Taken together, these observations suggest increased arginase activity in HARV cultured islets of Langerhans. Since insulin was not altered in Study A, we hypothesize that the decrease in arginine concentration observed in the present studies was not sufficient to alter basal insulin secretion. However, if there were an increase in insulin resistance during spaceflight which was present during the postprandial state, an increased conversion of arginine to ornithine may modulate a decreased ability for compensatory insulin secretion. The lack of a significant increase in the amino acid citrulline, rules out a more active nitric oxide synthase vs arginase. Thus, nitric oxide production via arginine to citrulline conversion, is likely not greater in the HARV, and islet blood perfusion, vascular reactivity, or membrane integrity is in all likelihood not compromised. The equimolar increase in urea, however, would suggest that an increase in total urea cycle activity is not a plausible explanation for these data. Decreases in lean body mass, and altered amino acid utilization illustrate that nitrogenous metabolites are influenced by spaceflight (34-36). In ground-based studies, the reduction in lean body mass occurs concomitantly with altered glucose and insulin metabolism (21, 22, 29). At present there is no unified hypothesis to explain these observations, although it has been suggested that a loss of lean body mass and subsequent release of nitrogenous metabolites, may be related to an increase in insulin resistance (33). The present studies are the first to document decreased glucose utilization, concomitant with alterations in nitrogenous analyte utilization, respective to an in vitro microgravity model system, and thus, both support and extend the data and hypotheses of Stein (33-36).

The aggregation of isolated islets of Langerhans into ICAs was a serendipitous discovery. From the four experiments conducted herein, we observed that islet aggregation occurs within 15 hours of HARV culture. That cells will aggregate within the HARV is a well known phenomenon, and has been documented elegantly in a recent review (38). Islet transplantation research currently underway which utilizes a microgravity model system for cell assembly, is aimed at developing an islet beta cell / sertoli cell aggregate for transplantation in Type 1

diabetes (5). The present studies illustrate that approximately 1700 whole islets will aggregate into ICAs that measure approximately 3 mm in diameter. The importance of such a large tissue mass may be salient to the success of pancreatic islet transplantation as a cure for Type 1 diabetes mellitus. At present, purified islets of Langerhans have been transplanted intraportally into the liver of Type 1 patients, resulting in normalization of plasma glucose concentrations (40). The requirement for immunosuppression is contraindicated to effective islet function, as most immunosuppressive agents decrease insulin sensitivity. Sertoli-islet cell aggregates may provide an immunoprivileged graft. However, these cells will still need to be transplanted into a highly vascularized organ site such as the liver. The potential of ICAs lies in the possibility of creating a greater tissue mass, with potential vascularization, which can be transplanted into a readily accessible vascularized organ such as the kidney capsule. Such an approach, if combined with sertoli cell technology, would provide a graft that is immunoprivileged and allow for removal of the graft in the presence of rejection. Such options are not currently available for intraportal islet transplants, and at present, recipients of purified islets require lifelong immunosuppression. Such possibilities are encouraging for ultimate success in the search for a cure for Type 1 diabetes; a cure which ideally will ameliorate the need for daily exogenous insulin administration, and prevent the devastating secondary complications of this disease.

In conclusion, the present studies demonstrated alterations in LPS induced TNF- α production of pancreatic islets of Langerhans, favoring a lesser TNF-production in the microgravity model system HARV paradigm. We conclude that rat pancreatic islets are possible sources of TNF during exposure to bacterial LPS and that insulin secretion may be altered by LPS-induced TNF secretion. Our results are most interesting since TNF has been implicated as possibly playing a significant role in the obesity-related development of insulin resistance, glucose intolerance and non-insulin-dependent diabetes mellitus. If one proposes that an infectious agent or even increased levels of circulating lipoprotein or glucose could stimulate TNF secretion within the islet which then may alter insulin secretion, intriguing hypotheses could be developed as to the local production of islet-derived TNF and its action on islet cell function in the whole body. The present studies also illustrated that basal insulin secretion was suppressed concomitant with an increase in TNF- α , and may be implicated in hormonal alterations of

spaceflight and the pathophysiology of Type 2 diabetes. Fuel homeostasis appears to be different in the HARV culture, as virtually no glucose was utilized in this paradigm, suggesting an alternative fuel source for ICAs. While nitrogenous compound analysis indicated a ubiquitous reliance upon glutamine in all experimental groups, arginine was converted to ornithine at a two-fold greater rate in the islets cultured in the HARV microgravity model system. Such observations suggest cell-specific effects of this system upon nitrogenous compound utilization. Finally, islets which are cultured in HARVs will aggregate into ICAs. A limited number of biochemical and physiological characteristics of these aggregates have been documented in the present studies. The clinical significance of these observations to the pathophysiology of diabetes or their relation to manned space flight, however, remains to be determined.

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Table 1. Nitrogenous metabolite concentrations (mmoles/ml) measured during the initial basal period of cell culture in HARV and Static Plates.

	0 Hrs		48 Hrs		Delta		Repeated ANOVA				
	HARV	PLATE	HARV	PLATE	HARV	PLATE	Between		Within		
							METHOD	0 Hr	48 Hr	TIME	TIME * METHOD
<i>Urea Cycle Metabolites</i>											
ARG	0.3431± 0.2050	0.3312± 0.0219	0.2529± 0.0187	0.2963± 0.0218	-0.0903± 0.0116	-0.0350± 0.0108	NS	NS	0.0001	0.0027	
ORN	0.0278± 0.0016	0.0264± 0.0046	0.1274± 0.0050	0.0752± 0.0051	0.0996± 0.0042	0.0488± 0.0049	NS	0.0001	0.0001	0.0001	
UREA	0.5508± 0.0227	0.5367± 0.0321	0.6764± 0.0239	0.6058± 0.0262	0.1256± 0.0134	0.0690± 0.0252	NS	NS	0.0001	NS	
NH ₃	0.1572± 0.0092	0.1559± 0.0102	0.3574± 0.0179	0.3238± 0.0178	0.2002± 0.0207	0.1679± 0.0176	NS	NS	0.0001	NS	
<i>Nitrogen Transfer Metabolites</i>											
GLU	0.9748± 0.0673	0.9311± 0.0713	1.0152± 0.0657	0.9826± 0.0666	0.0403± 0.0227	0.0515± 0.0365	NS	NS	0.0469	NS	
GLN	0.7643± 0.0266	0.7285± 0.0374	0.6516± 0.0146	0.6557± 0.0219	-0.1127± 0.0216	-0.0729± 0.0279	NS	NS	0.0001	NS	
ALA	0.7212± 0.336	0.6936± 0.0392	0.7620± 0.0289	0.7378± 0.0303	0.0408± 0.0175	0.0442± 0.0291	NS	NS	0.0223	NS	
<i>Neurotransmitter Synthesis</i>											
GABA	0.0065± 0.0018	0.0063± 0.0017	0.0277± 0.0043	0.0293± 0.0069	0.0212± 0.0032	0.0230± 0.0058	NS	NS	0.0001	NS	
PSER	0.0096± 0.0078	0.0090± 0.0078	0.0144± 0.0015	0.0123± 0.0011	0.0048± 0.0009	0.0033± 0.0006	NS	NS	0.0001	NS	
<i>Creatine, Heme, Purine Synthesis</i>											
GLY	0.7800± 0.0389	0.7462± 0.0437	0.8187± 0.0333	0.7897± 0.0351	0.0387± 0.0192	0.0435± 0.0303	NS	NS	0.0341	NS	

Data are means ±SEM for 10 islet populations per cell. Delta=48 hr value - 0hr value. A two-way between-within ANOVA with interaction was used to differentiate influences of culture METHOD (HARV vs. PLATE) vs. TIME (0hr vs. 48hr). NS, not significant at p<0.05. LEU, TYR, PHE, BALA, ETN, LYS, HIS, TAU, ASP, HYP, THR, SER, ASN, PRO, CIT, CYC, VAL, MET, HCY, and ILE were not significantly different (p>0.05). BABA, HCYS, TRP, HYL/AHY, IMHIS, 3-MHIS, ASN, CARN, PETN, GA, SAR, AAD, and ABU were not detectable in the analysis.

Table 2. *Lactate Dehydrogenase activity (LDH) measured during 48 hours of cell culture in HARV and Static Plates, with and without LPS stimulation.*

	Hours Post LPS Stimulation					
	0	3	6	12	24	48
HARV-CN	0.14±0.11	0.12±0.01	0.14±0.01	0.12±0.01	0.15±0.02	0.14±0.01
HARV-LPS	0.14±0.01	0.13±0.02	0.13±0.02	0.12±0.01	0.16±0.02	0.13±0.02
PLATE-CN	0.15±0.01	0.14±0.02	0.15±0.01	0.14±0.01	0.17±0.02	0.15±0.01
PLATE-LPS	0.16±0.01	0.17±0.01	0.16±0.01	0.13±0.00	0.23±0.02	0.14±0.01

Data are means ±SEM for 3 separate islet populations per mean. Between-subjects ANOVA indicated no differences between groups, at any time point ($p>0.05$). Overall experiment analysis with all time points considered indicated less LDH in HARV vs. PLATE cultures ($p=0.0505$). Within-subjects effects were significant only for TIME ($p=0.001$).

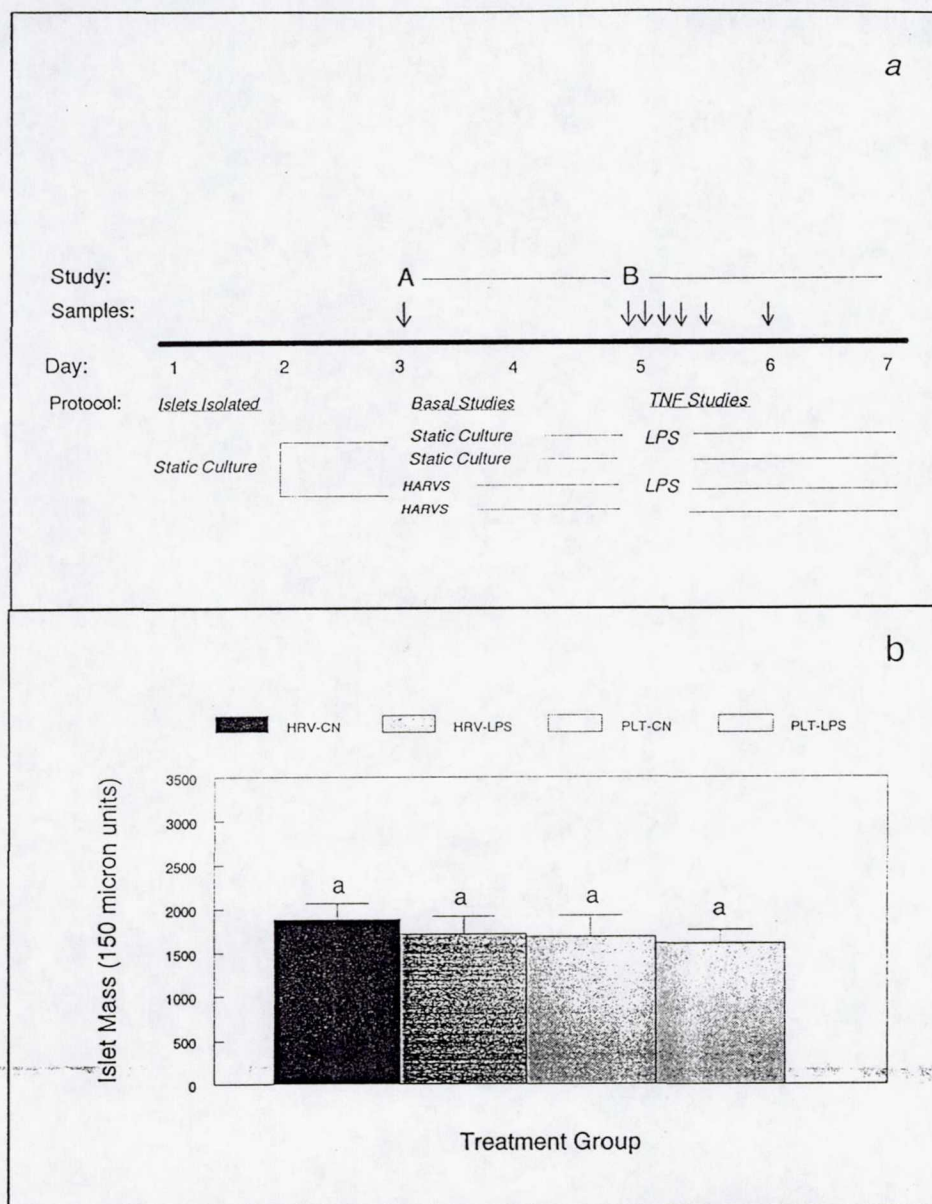


FIGURE 1. The experimental design (1a) and the islet equivalent units per treatment group (1b). HRV-CN, HARV control; HRV-LPS, HARV LPS-stimulated; PLT-CN, static culture plate control; PLT-LPS, static culture plate stimulated with LPS. Data are means \pm SEM.

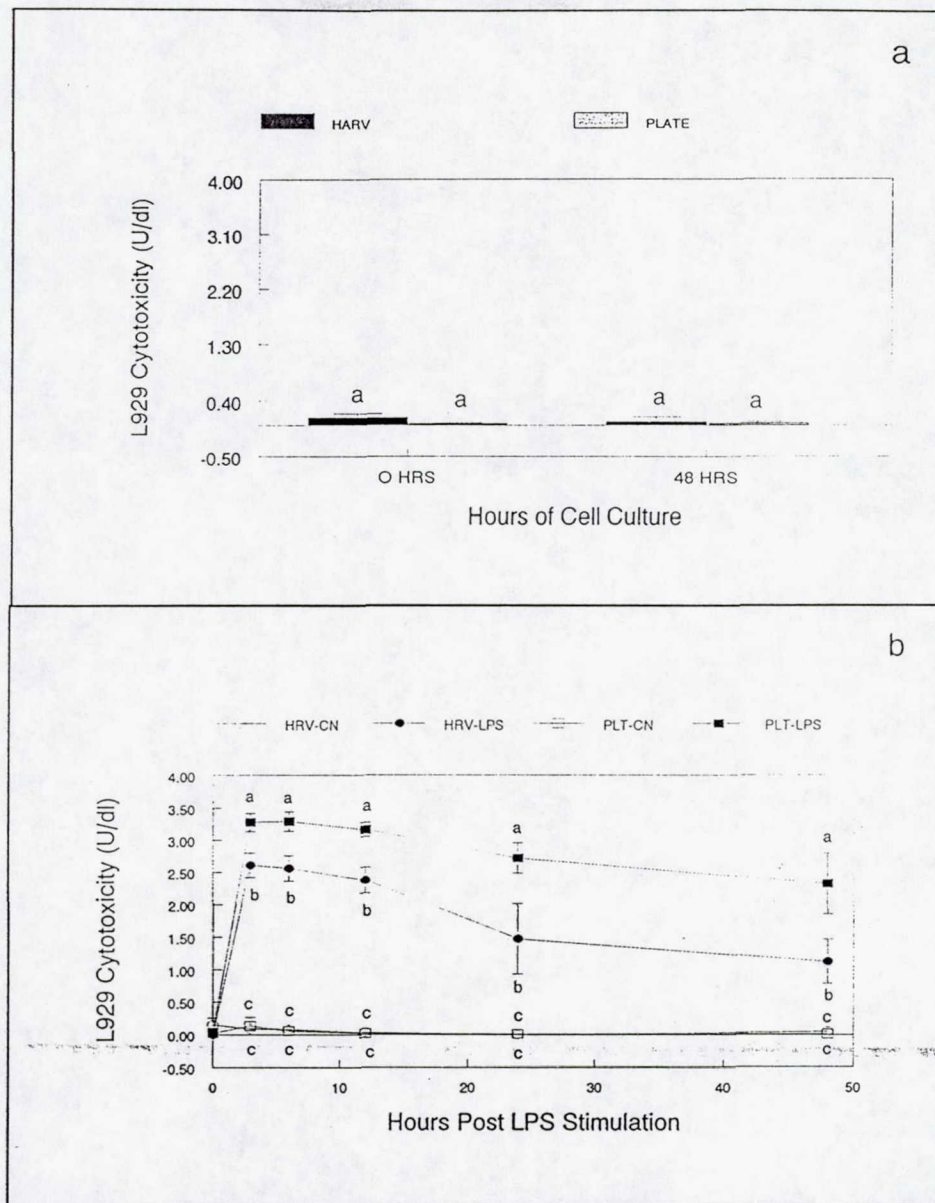


FIGURE 2. TNF-alpha in islet medium: a) during 48 hours culture, b) post LPS stimulation, HRV-CN, HARV control; HRV-LPS, HARV LPS-stimulated; PLT-CN, static culture plate control; PLT-LPS, static culture plate stimulated with LPS. Data are means \pm SEM. There were no significant differences in L929 TNF-alpha cytotoxicity for any treatment group Study A. Following stimulation with LPS there were significant increases in TNF-alpha (Study B). Significant between-subjects effects were noted for METHOD ($p=0.0049$, static culture > HARV), LPS ($p=0.0001$, LPS > non-LPS), and HARV*LPS ($p=0.0046$, static culture LPS > HARV LPS). Within-subjects effects were significant for TIME ($p=0.0001$) and interaction of HOURS*LPS ($p=0.0001$).

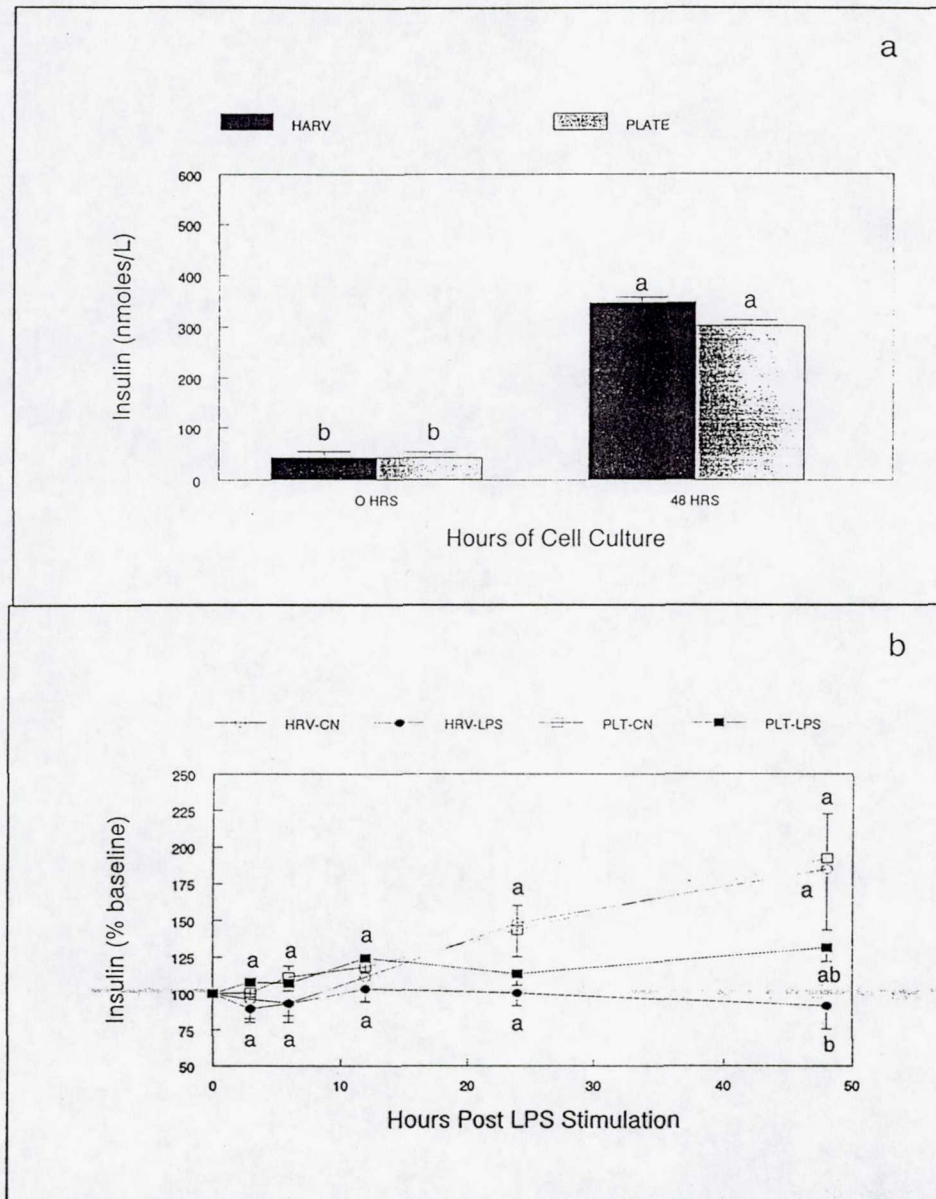


FIGURE 3. Insulin in islet medium: a) during 48 hours culture, b) post LPS stimulation. HRV-CN, HARV control; HRV-LPS, HARV LPS-stimulated; PLT-CN, static culture plate control; PLT-LPS, static culture plate stimulated with LPS. Data are means \pm SEM. At time zero, insulin values were not different in Study A, however, ANOVA indicated a significant effect of TIME ($p=0.0004$). In Study B, insulin concentration increased in both HARV and static culture islets not stimulated with LPS. There were no significant differences between-subjects; however, within-subjects effects of HOURS and HOURS*LPS were significant ($p=0.0001$, $p=0.0001$). In both LPS-stimulated cultures insulin secretion was less compared to controls. At 48 hours both control groups were different from the HARV-LPS group; the static plate culture islets with LPS was intermediate in insulin content.

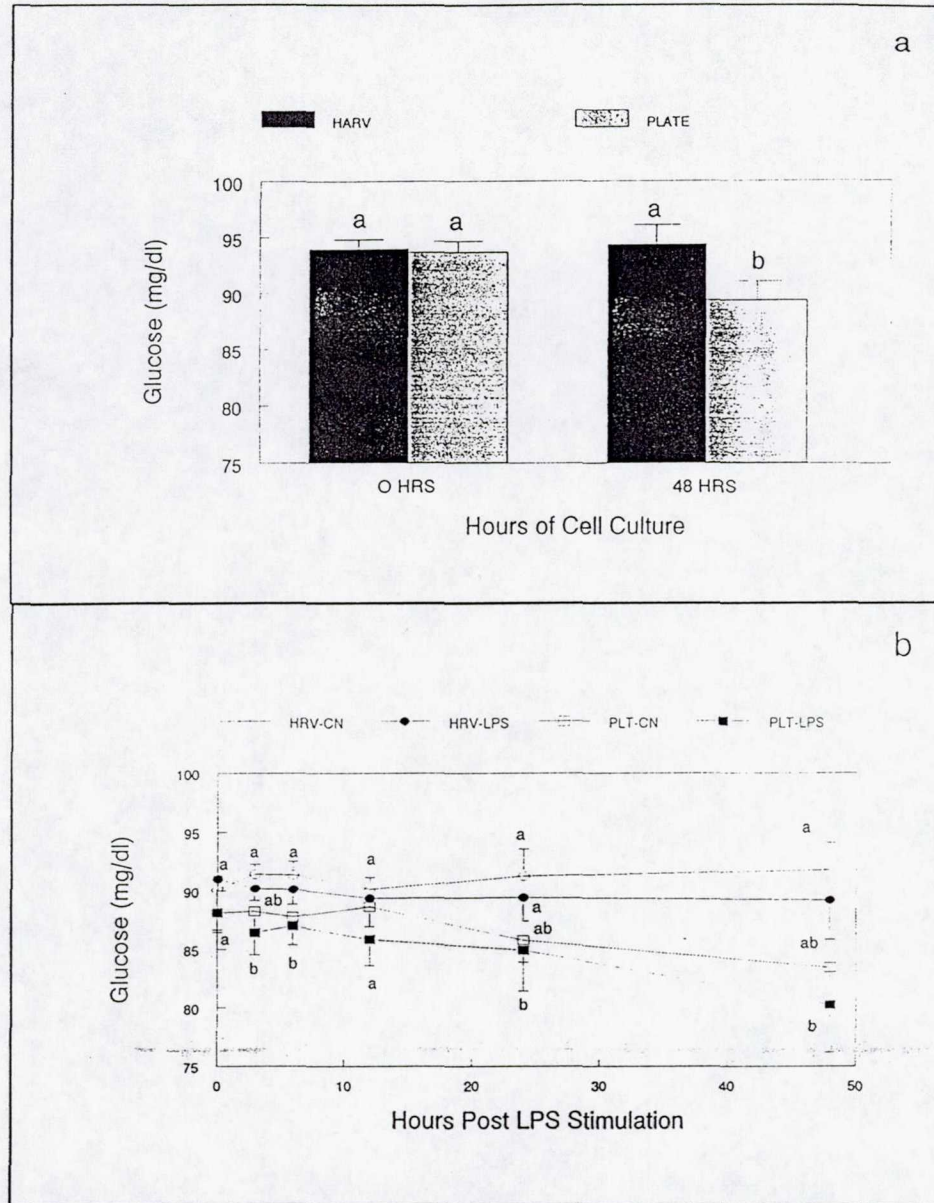


FIGURE 4. Glucose in islet medium: a) during 48 hours culture, b) post LPS stimulation. HRV-CN, HARV control; HRV-LPS, HARV LPS-stimulated; PLT-CN, static culture plate control; PLT-LPS, static culture plate stimulated with LPS. Data are means \pm SEM. At time zero, there were no significant differences in the glucose concentration in islet medium in Study A; however, following 48 hours, there was a significant interaction of Time and Method ($p=0.0488$). The media from islets in the PLATE culture had the lowest glucose concentration at 48 hours ($p<0.05$). In Study B, there were minor differences in the media glucose concentration up to 12 hours of culture. ANOVA indicated a significant effect of METHOD ($p=0.0203$, HARV > static culture) and HOURS ($p=0.0026$). At 48 hours post LPS stimulation, the static culture group had metabolized the greatest amount of glucose from the media. The highest media glucose concentrations were observed in the non LPS-stimulated HARV cultures, which were significantly different from the LPS stimulated static culture islets.

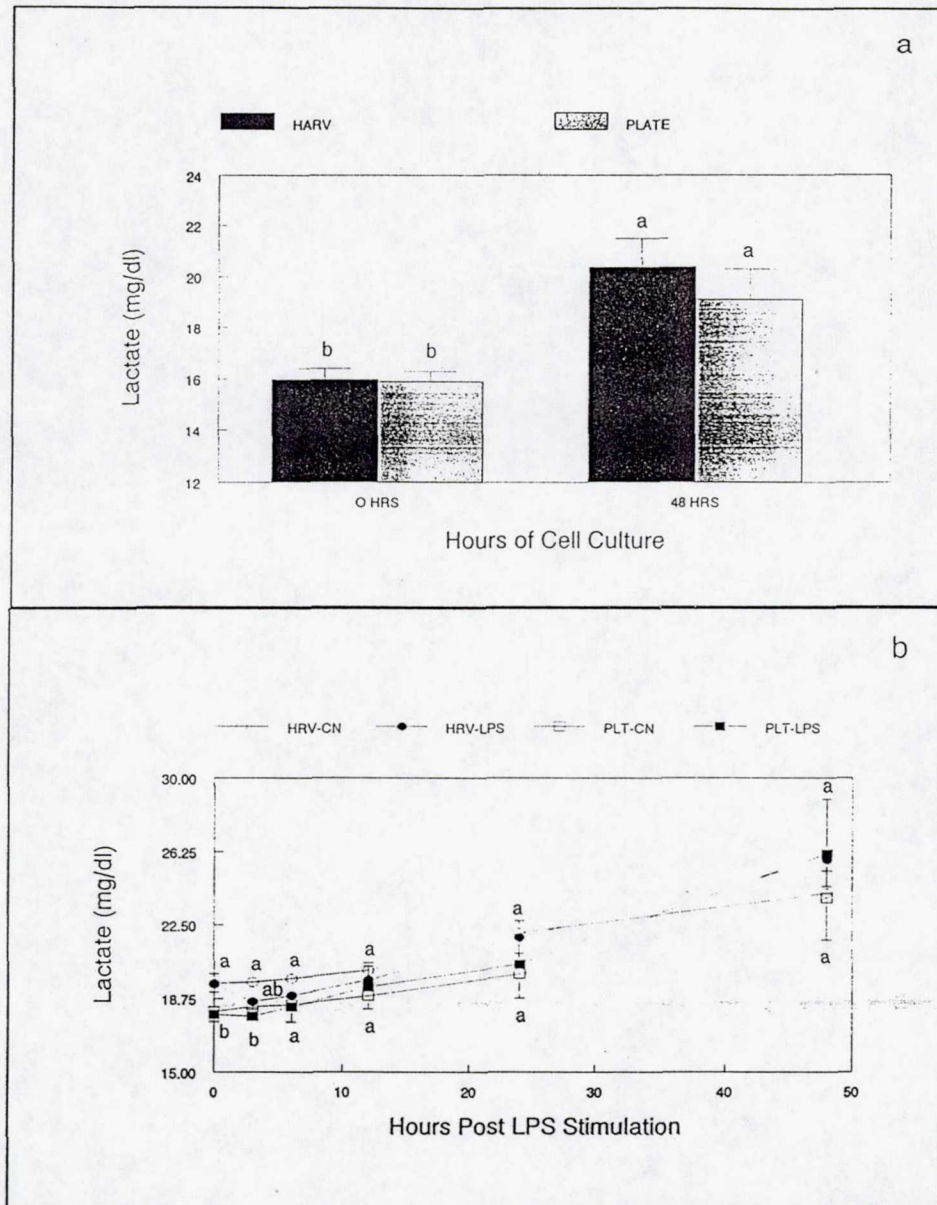


FIGURE 5. Lactate in islet medium: a) during 48 hours culture, b) post LPS stimulation. HRV-CN, HARV control; HRV-LPS, HARV LPS-stimulated; PLT-CN, static culture plate control; PLT-LPS, static culture plate stimulated with LPS. Data are means \pm SEM. The 48 hour value was significantly different from the zero hour value as the repeated measures ANOVA illustrated a significant effect of TIME in Study A ($p=0.0001$, 48 HRS > 0 HRS). During LPS stimulation (Study B) all treatment groups increased their lactate concentrations; ANOVA main effects were significant only for TIME ($p=0.0001$).

a

b

FIGURE 6. Phase-contrast photomicrographs of freshly isolated islets of Langerhans from male Wistar Furth rats (6a), and islets cultured for 48 hours in the HARV (6b). All images were obtained using a 40x air objective with a green filter and white fiber optic side illumination.